Phosphatidylinositol 3-kinase binding to polyoma virus middle tumor antigen mediates elevation of glucose transport by increasing translocation of the GLUT1 transporter

(signal transduction/neoplastic transformation/vesicular transport)

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ABSTRACT Elevation in the rate of glucose transport in polyoma virus-infected mouse fibroblasts was dependent upon phosphatidylinositol 3-kinase (PI 3-kinase; EC 2.7.1.137) binding to complexes of middle tumor antigen (middle T) and pp60[c-src]. Wild-type polyoma virus infection led to a 3-fold increase in the rate of 2-deoxyglucose (2DG) uptake, whereas a weakly transforming polyoma virus mutant that encodes a middle T capable of activating pp60[c-src] but unable to promote binding of PI 3-kinase induced little or no change in the rate of 2DG transport. Another transformation-defective mutant encoding a middle T that retains functional binding of both pp60[c-src] and PI 3-kinase but is incapable of binding Shc (a protein involved in activation of Ras) induced 2DG transport to wild-type levels. Wortmannin (≤100 nM), a known inhibitor of PI 3-kinase, blocked elevation of glucose transport in wild-type virus-infected cells. In contrast to serum stimulation, which led to increased levels of glucose transporter 1 (GLUT1) RNA and protein, wild-type virus infection induced no significant change in levels of either GLUT1 RNA or protein. Nevertheless, virus-infected cells did show increases in GLUT1 protein in plasma membranes. These results point to a posttranslational mechanism in the elevation of glucose transport by polyoma virus middle T involving activation of PI 3-kinase and translocation of GLUT1.

The polyoma virus middle tumor antigen (middle T) is responsible for most if not all of the cellular changes that accompany neoplastic transformation of established cells in culture and is essential for the induction of a broad spectrum of tumors in mice (1, 2). Middle T is a membrane-bound protein devoid of enzymatic activity but able to promote the formation of multienzyme complexes resembling those of activated growth factor receptors. Middle T expression leads to morphological changes and loss of cell growth control (2–5). Among the discrete physiological changes linked to neoplastic transformation and dependent on middle T are elevated rates of glucose transport (4, 6), induction of ribosomal protein S6 phosphorylation (7), and increased expression of early response genes (8–10). Binding of middle T to pp60[c-src] activates the latter's protein tyrosine kinase activity, which results in the phosphorylation of middle T on specific tyrosines. Phosphorylation of middle T on Tyr-315 creates a Src homology 2 (SH2)-binding site for the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase; EC 2.7.1.137) (11–13). Phosphorylation of middle T on Try-250 creates a binding site for Shc (14, 15), a Grb2-mSos1 binding protein involved in activation of Ras (16).

Studies of polyoma virus mutant 315YF, encoding a middle T in which Tyr-315 is replaced by Phe (17), have shown that this altered middle T activates pp60[c-src] normally but fails to promote binding of PI 3-kinase (11, 18). The 315YF mutation significantly diminishes the ability of the virus to transform established rat fibroblasts (17) as well as its ability to induce tumors when inoculated into newborn mice (19). It is therefore important to understand the downstream consequences of PI 3-kinase binding to middle T and its role in altering physiological properties of the cell.

Elevation in the rate of glucose transport occurs in response to a number of different hormones, growth factors, and oncogenes. Glucose transport is mediated by a family of at least five tissue-specific glucose transporters designated GLUT1 through GLUT5 (20, 21), with GLUT1 being recognized as the principal transporter in fibroblasts. A variety of mechanisms leading to increased rates of glucose transport have been described, including transcriptional activation of transporter genes, stabilization of transporters, and increased translocation of transporters from intracellular vesicles to the plasma membrane (20, 22, 23). In this investigation, wild-type polyoma virus infection was found to elevate glucose transport through a posttranslational mechanism involving PI 3-kinase and GLUT1 translocation.

MATERIALS AND METHODS

Viruses and Cells. Polyoma virus stocks were prepared from infected primary baby mouse kidney (BMK) cells (24). To remove residual growth factors in crude virus lysates, virus was pelleted by centrifugation at 80,000 × g for 2 h and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 0.01% bovine serum albumin (BSA; Sigma, fraction V). Throughout this study the RA strain of polyoma virus (25) was used as the wild-type virus. Polyoma viruses NG-59 (26) and 315YF (originally called 1178T) (17) have been previously described. Polyoma virus 250YS is a weakly transforming mutant encoding a middle T containing Ser in place of Tyr-250 (J.D., unpublished work), the site of phosphorylation required for binding Shc (14, 15). NIH 3T3 cells grown on 12-well cluster plates were mock infected with BMK extract or infected with polyoma virus for 1.5 h, washed twice with 1 ml of phosphate-buffered saline containing 1 mM MgCl$_2$ and 1 mM CaCl$_2$ (PBS), and serum starved for 36–40 h in DMEM containing 0.01% BSA. Multiplicities of infection were over a range of approximately 2–50 plaque-forming units per cell.

Rat F111 clones stably expressing NG-59, 315YF, or wild-type middle T were isolated by G418 selection of cells infected with retroviruses containing middle T genes (27). pZIPNeoSV(X)1 DNA containing the NG-59 middle T gene was provided by T. Roberts (Dana–Farber Cancer Institute, Boston).

Northern Blot Analysis. RNA was prepared essentially as described (28). RNA electrophoretically resolved on formaldehyde gels and transferred to nylon membranes (Stratagene Duralon-UV membranes) was probed with ^{32}P-labeled random primers (BRL random primer kit) derived from plasmid prGT4-12, which contains a 2.6-kb GLUT1 DNA insert (29), or plasmid MAT1.1, which contains a mouse α-tubulin gene (provided by B. Neel, Harvard Medical School). Hybridization was performed in 5× SSC/50% formamide/1% SDS/2× Denhardt's solution/0.5% nonfat dry milk at 42°C. Membranes were washed twice for 15 min at room temperature in 1× SSC/0.1% SDS and twice for 15 min at room temperature in 0.25% SSC/0.1% SDS and were autoradiographed with an intensifying screen at −80°C.

2-Deoxyglucose (2DG) Transport Assay. The rate of glucose transport was determined by incorporation of [^{3}H]2DG (New England Nuclear, 30–60 Ci/mmol; 1 Ci = 37 GBq) into serum-starved cells 36–40 h after infection. Cells were preincubated for 30 min in 1 ml of PBS/0.1% glucose, washed twice with 1 ml of PBS, and incubated for 30 min at 37°C in 1 ml of PBS/100 μM 2DG containing 2.5 μCi of [^{3}H]2DG. Rates of incorporation were linear up to 1 h. Reactions were terminated by washing twice with 2 ml of PBS and adding 0.5 ml of 1% SDS. Extracts were quantitated for 2DG incorporation by scintillation counting, protein levels by BCA (bicinchoninic acid) assay (Pierce), and middle T by phosphorescence imaging (Bio-Rad) analysis of Western blots. Wortmannin (Sigma), stored in dimethyl sulfoxide, was diluted in DMEM just prior to use.

Quantitation of Middle T, PI 3-Kinase, and pp60^{c-src} Protein Tyrosine Kinase Activities. PI 3-kinase and protein tyrosine kinase assays were performed on immunoprecipitated tumor (T) antigens as described (11) except that PI 3-kinase reactions were conducted at 30°C and in the presence of phosphatidylserine (0.2 mg/ml). Middle T Western blots were prepared with anti-T monoclonal antibody F4 (30). Anti-Shc Western blots of middle T immunoprecipitated with PAb762 (provided by S. Dilworth, Hammersmith Hospital, London) were prepared with rabbit anti-Shc sera (Transduction Labs, Lexington, KY) and ^{125}I-labeled second antibody (ICN) or enhanced chemiluminescence (ECL; Amersham).

Immunofluorescence of Epitope-Tagged GLUT1. P2 cells were used to examine plasma membrane GLUT1. P2 cells were derived from NIH 3T3 cells by transfection of a plasmid encoding an epitope-tagged GLUT1 in which the first extracellular loop was replaced by the corresponding domain of rat GLUT4, into which the insulin receptor P2 epitope (RDIYET-DYYRKGGKGLLPVR) was inserted (K. Morioka, K. J. Verhey, and M.J.B., unpublished work). Epitope tagging of this loop in GLUT1 and GLUT4 has been shown not to affect transport function (31) or subcellular localization (31, 32). Polyoma virus-infected P2 cells, grown on polylysine-coated coverslips, were fixed in 3% paraformaldelyde/PBS. Plasma membrane GLUT1 was detected by using affinity-purified polyclonal rabbit antibody raised against the insulin receptor-P2 peptide and fluorescein-conjugated goat anti-rabbit antibody (Cappel). Cells were fixed again, permeabilized with 0.2% Triton X-100/PBS (10 min), and stained for T antigen with rat anti-T antigen ascites fluid and rhodamine-conjugated goat anti-rat antibody (Jackson ImmunoResearch).

RESULTS

Elevation of Glucose Transport in Polyoma Virus-Infected Cells Requires Binding of PI 3-Kinase to Middle T. Previous work has established that some aspect of middle T function is

required for elevation of glucose transport in virus-infected cells (4). Middle T-stimulated transport involves a stereospecific transporter(s), since incorporation of 2DG is inhibitable by D-glucose or cytochalasin B (a known inhibitor of glucose transporters) but is unaffected by L-glucose (data not shown).

The requirement for binding of PI 3-kinase to middle T in elevation of glucose transport is apparent from studies of middle T mutants altered in sites of phosphorylation by pp60^{c-src}. Mutants 315YF and 250YS are defective in PI 3-kinase and Shc binding, respectively. Both mutants induce some degree of morphological transformation of rat fibroblasts but are unable to induce growth in soft agar. Mutant 315YF has been tested in newborn mice and found to be compromised in its ability to induce tumors (19). NG-59, an hr-t (host range transformation-defective) mutant, is totally defective in all aspects of transformation and in all middle T-linked biochemical functions, including activation of pp60^{c-src} (26, 33, 34). These mutants were used along with wild-type virus to investigate the mechanism of elevation of glucose transport by polyoma middle T.

2DG transport in polyoma virus-infected NIH 3T3 cells was measured at about 36 h after infection, prior to development of cytopathic effects. Cultures infected in parallel were extracted at the same time to assay for amounts of middle T and associated proteins. Rates of transport are plotted against different levels of middle T, achieved by varying the multiplicities of infection. As shown in Fig. 1 *a* and *b*, wild-type virus infection resulted in elevated rates of 2DG uptake, up to 3-fold over mock-infected cells. Mutants NG-59 and 315YF were both impaired in their ability to elevate transport, even under conditions where they express 2- to 3-fold more middle T protein than the wild-type virus. Similar results with wild-type and mutant viruses were obtained when BALB/3T3 cells were used. The slight elevation in 2DG transport seen at higher levels of 315YF or NG-59 mutant middle T expression does not necessarily indicate an effect of the altered middle T proteins but is rather a consequence of virus infection, since a mutant which expresses no middle T at all also had a small effect (ref. 4; A.T.Y., unpublished data). In contrast to mutants NG-59 and 315YF, mutant 250YS induced 2DG transport fully as well as did wild-type virus (Fig. 1b). The induction of glucose transport by polyoma virus appears to approach saturation at higher multiplicities of infection, since expression of wild-type or 250YS middle T beyond a certain level leads to little further elevation in the rate of 2DG uptake (Fig. 1b).

Levels of middle T expression and binding of middle T-associated proteins are shown in Fig. 1 *c–f*. The results show that the binding of PI 3-kinase but not of Shc to middle T is required for elevation of 2DG transport. Results with mutant 315YF in particular make it clear that PI 3-kinase binding and not just pp60^{c-src} activation by middle T is required. Results with the weakly transforming mutant 250YS further demonstrate that PI 3-kinase binding and induction of glucose transport are not sufficient for complete transformation.

The effects of wild-type and mutant middle T proteins on glucose transport were also examined in clones of established rat embryo fibroblasts transduced by middle T-encoding retroviruses. As seen in Table 1, expression of wild-type middle T but not NG-59 or 315YF middle T significantly raised the 2DG transport rate. These results, found in a number of different clonal isolates, demonstrate the requirement for PI 3-kinase binding under conditions of stable expression as well as in lytic virus infection.

Wortmannin Blocks Elevation of 2DG Transport in Wild-Type Virus-Infected Cells. As a further test of an essential role for PI 3-kinase in polyoma virus-induced elevation of glucose transport, wild-type virus-infected cells were incubated in the presence of wortmannin, a known inhibitor of PI 3-kinase (35, 36). The results show that wortmannin at submicromolar concentrations blocked the elevation of glucose transport (Fig.
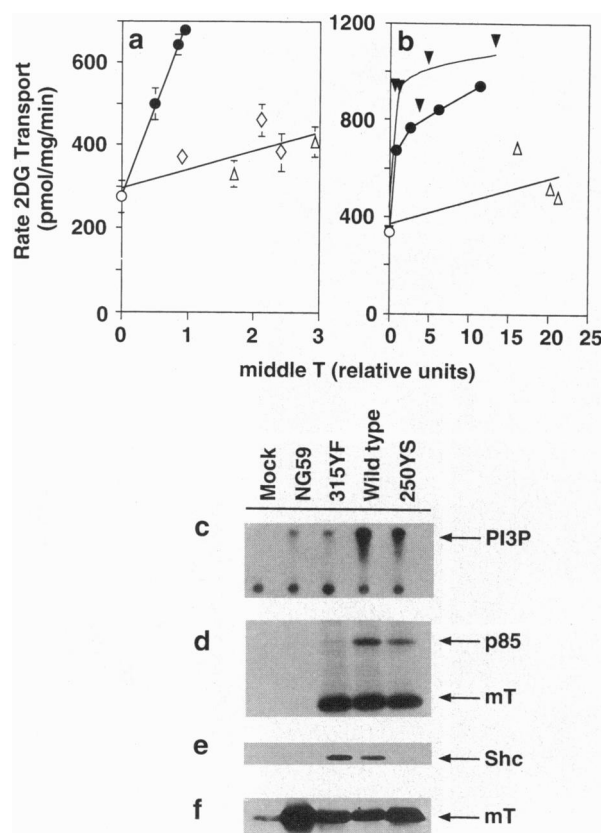
FIG. 1. Polyoma virus induction of glucose transport is impaired when middle T fails to bind PI 3-kinase. (*a* and *b*) Rate of 2DG transport in polyoma virus-infected NIH 3T3 cells plotted against the relative levels of middle T. Cells were infected with serial dilutions of virus to create a range of middle T expression. (*a*) 2DG incorporation in mock- (O), NG-59- (△), 315YF- (◇), or wild-type polyoma virus- (●) infected cells (mean of triplicate determinations ± SD). (*b*) 2DG incorporation in mock- (O), NG-59- (△), 250YS- (▼), or wild-type polyoma virus- (●) infected cells. (*c–f*) Lysates of polyoma virus-infected cells were tested for PI 3-kinase activity (PI3P, phosphatidylinositol 3-phosphate) in T-antigen immunoprecipitates (*c*), protein tyrosine kinase activity in T-antigen immunoprecipitates (*d*), p52 Shc in middle T (mT) immunoprecipitates (*e*), and middle T by Western blotting (*f*).

2*a*). Over the same concentration range and in a roughly parallel fashion, wortmannin also inhibited middle T-associated PI 3-kinase *in vivo* (Fig. 2*b*). The inhibitor did not affect either the level of expression of middle T or the binding of the p85 subunit of PI 3-kinase to middle T (data not shown).

Differences in the Mechanisms of Action of Polyoma Virus and Serum in Increasing Glucose Transport. Serum and growth factors elevate glucose transport by transcriptionally activating the GLUT1 gene (20). The v-*src* (37) and v-*fps* (38) oncogenes transcriptionally activate GLUT1 in rodent fibro-
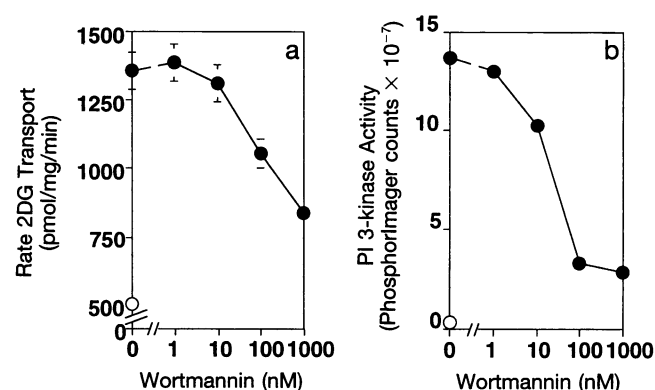

FIG. 2. Wortmannin inhibits polyoma virus-induced elevation of glucose transport and middle T-associated PI 3-kinase *in vivo*. Mock- (O) or wild-type polyoma virus- (●) infected NIH 3T3 cells were serum starved for 20 h and tested for the rate of [³H]2DG uptake (*a*) or middle T-associated PI 3-kinase activity (*b*). One hour prior to transport assay or detergent extraction for T-antigen immunoprecipitation, the indicated concentrations of wortmannin were added to cells. PI 3-kinase assays were performed on extracts without added wortmannin.

blasts, and v-*src*, in chick fibroblasts, acts to stabilize GLUT1 protein (23). Since middle T activates the tyrosine kinase activity of pp60[c-src], GLUT1 Northern and Western blots were prepared from wild-type polyoma virus-infected cells. No increases in GLUT1 RNA or protein were detectable in NIH 3T3 cells infected with wild-type virus (Fig. 3). Increases in GLUT1 RNA and protein were readily detected 5 h after serum stimulation, as previously reported (39). To rule out the possibility that a glucose transporter other than GLUT1 might contribute to the overall rates of 2DG uptake, cells were examined for the presence of GLUT3 and GLUT4 by Western blotting. These transporters were not detected in uninfected, infected, or serum-stimulated NIH 3T3 cells. GLUT2 and GLUT5 are not known to be expressed in fibroblasts.

These results show that, in contrast to stimulation with serum, polyoma virus infection elevates glucose transport without measurably inducing or altering total levels of glucose transporters. The virus thus appears to exert an effect at a posttranslational level, possibly involving increased translocation of transporter molecules from the cytoplasm to the plasma membrane, or by some effect on the activity of plasma membrane transporters *per se*.

Serum stimulation of glucose transport in 3T3 cells has been shown to be biphasic, involving a rapid response that occurs within 30–40 min and in the absence of protein synthesis, and a slower response that is inhibitable by cycloheximide (40). The immediate response appears to be posttranslational and, as with the virus, may well be linked to PI 3-kinase through any of a number of growth factor receptor tyrosine kinases. The major component of the serum response which follows is clearly at the transcriptional level and involves *de novo* syn-

Table 1. Rate of 2DG transport in rat F111 cells stably expressing NG-59, 315YF, or wild-type middle T genes

Cells	Rate of 2DG transport	
	pmol/min per mg of protein	-fold
*F*Neo	311.2 ± 31.2	1.0
*F*NG-59	402.4 ± 30.0	1.3
*F*315YF	555.8 ± 29.7	1.8
*F*WTmT	1324.0 ± 291.4	4.3

Clones of rat F111 cells transduced with vector alone (*F*Neo), NG-59 middle T (*F*NG-59), 315YF middle T (*F*315YF), or wild-type middle T (*F*WTmT) genes were tested for the rate of 2DG transport. Results are the mean of triplicate determinations ± SD.
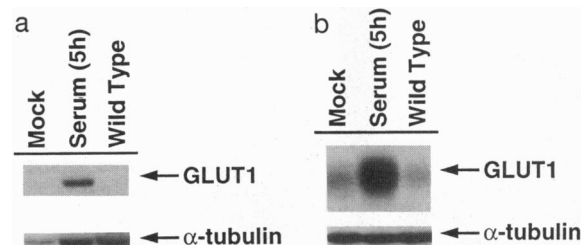

FIG. 3. GLUT1 RNA and protein in polyoma virus-infected cells. NIH 3T3 cells were either mock-infected or infected with wild-type polyoma virus and serum starved for 30 h. For serum-stimulated mock-infected cells, 15% calf serum was added 25 h after infection. (*a*) Northern blot of GLUT1 and α-tubulin control mRNAs. (*b*) Western blots.

thesis of GLUT1. In support of the idea that the virus acts posttranslationally and serum acts both pre- and posttranslationally, 2DG transport in virus-infected cells was further elevated by addition of serum (Fig. 4).

Wild-Type Polyoma Virus Infection Leads to Increased Translocation of GLUT1 to the Plasma Membrane. To determine directly whether wild-type polyoma virus infection leads to increased translocation of GLUT1, a double immunofluorescence study was performed on polyoma virus-infected P2 cells which express an epitope-tagged GLUT1 (see *Materials and Methods*). Results on 2DG uptake in virus-infected P2 cells were essentially the same as described above for NIH 3T3 cells. Infected P2 cells were first fixed but not permeabilized and stained for surface GLUT1, and then permeabilized and stained for nuclear T antigen. As shown in Fig. 5, wild-type virus and mutant 250YS induced elevated amounts of plasma membrane GLUT1 in 50–60% of infected cells, while mutants NG-59 and 315YF had little effect over the background seen in uninfected cells. To exclude the possibility that the anti-P2 antibody was detecting intracellular GLUT1, cells were also stained for α-tubulin. Little or no α-tubulin staining was detected under conditions used to detect surface GLUT1 (result not shown). These results indicate that wild-type polyoma virus infection induces increased translocation of GLUT1 to the plasma membrane and that middle T–PI 3-kinase binding is needed for this translocation to occur.

DISCUSSION

PI 3-kinase binding to middle T–pp60[c-src] was shown to mediate the elevation in rate of glucose transport in polyoma virus-infected and transformed cells. Nontransforming or weakly transforming virus mutants encoding middle Ts which are defective in PI 3-kinase binding were impaired in their ability to elevate glucose transport. In contrast, a weakly transforming mutant encoding a middle T capable of binding PI 3-kinase but defective in binding Shc elevated transport to wild-type levels. The PI 3-kinase inhibitor wortmannin effectively blocked elevation of 2DG transport by wild-type virus, lending further support to the conclusion that the effect on transport by polyoma middle T is mediated through PI 3-kinase. While it is possible that another SH2-containing adaptor protein such as Nck, reported to bind the platelet-derived growth factor (PDGF)-β receptor at the same site as PI 3-kinase (41), mediates middle T-induced glucose transport, we have been unable to detect Nck in wild-type middle T immunoprecipitates by Western blotting (data not shown).

While serum transcriptionally activates the GLUT1 gene, polyoma virus middle T and PI 3-kinase act at a posttranslational level involving translocation of intracellular GLUT1. It appears that virus-infected serum-deprived NIH 3T3 cells operate at a near-maximal rate of transport achieved by translocation
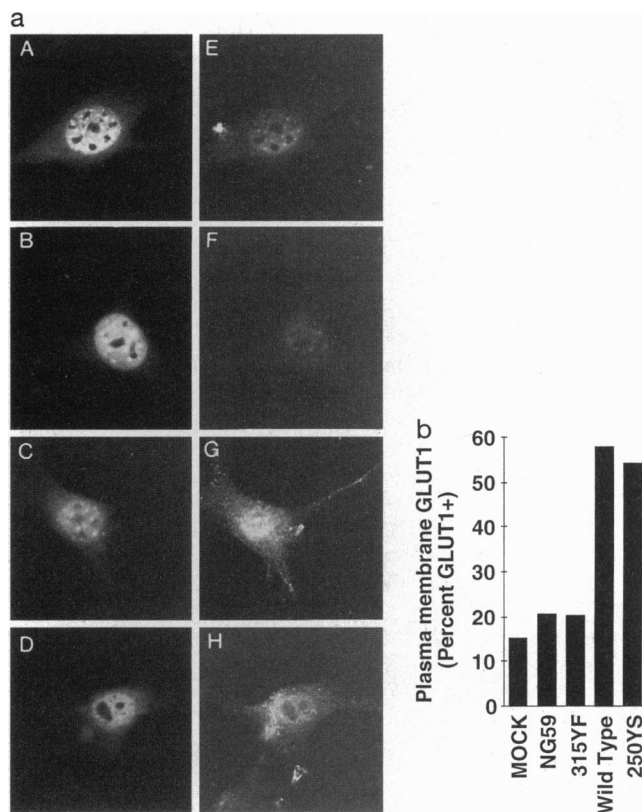
FIG. 5. Indirect immunofluorescence of plasma membrane GLUT1 in polyoma virus-infected P2 cells. NIH 3T3 cells expressing an epitope-tagged GLUT1 (P2 cells) were infected with polyoma viruses NG-59, 315YF, 250YS, or wild type and serum starved. Epitope-tagged GLUT1 was detected on the cell surface by staining of paraformaldehyde-fixed cells. After GLUT1 staining, cells were refixed and permeabilized with Triton X-100 and stained for T antigen. (*a*) T-antigen (*A–D*) and epitope-tagged GLUT1 (*E–H*) staining of P2 cells infected by polyoma virus NG-59 (*A* and *E*), 315YF (*B* and *F*), wild type (*C* and *G*), and 250YS (*D* and *H*). (×1000.) (*b*) Percentage of cells with enhanced cell surface GLUT1 staining. Cell surface GLUT1 was scored in T-antigen-positive cells in all virus-infected cultures and in randomly selected cells in mock-infected cultures.

of available GLUT1 molecules to the plasma membrane. Addition of serum to such cells further elevates 2DG transport primarily by increasing *de novo* synthesis of GLUT1.

Binding of PI 3-kinase to middle T activates its enzymatic activity (42), and levels of 3-phosphoinositides are known to be increased in cells expressing polyoma virus middle T (43, 44). Since these phospholipids are not turned over by phospholipase C to give rise to second messengers (45, 46), they may serve directly to signal events from the membrane. One or more of the 3-phosphoinositides may thus play a role in the translocation of GLUT1 from intracellular vesicles to the plasma membrane in polyoma virus-infected cells. This possibility is consistent with evidence for a role of PI 3-kinase in glucose transport and vesicular trafficking in other systems. In *Xenopus* oocytes wortmannin inhibits the stimulation of 2DG transport by insulin-like growth factor-1, while microinjection of a phosphopeptide that activates PI 3-kinase stimulates transport (47). In HepG2 cells, PI 3-kinase mediates endocytic trafficking of internalized platelet-derived growth factor receptors (48). Inhibitors of PI 3-kinase prevent insulin-stimulated elevation of glucose transport (49) and GLUT4 translocation (50) in 3T3 L1 cells. The yeast Vps34 protein is a PI 3-kinase required for sorting of vesicles to lysosome-like vacuoles (51). Interestingly, both Vps34 and a polyoma virus mutant similar to 250YS were shown to elevate levels of PI3P but not of the more highly phosphorylated 3-phosphoinositides (51, 52),
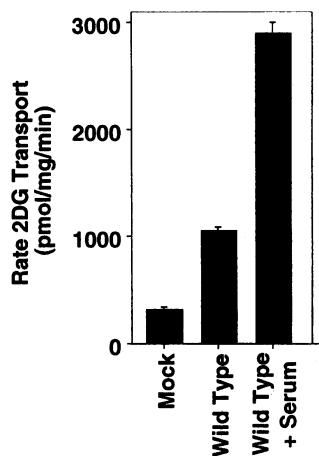

FIG. 4. Serum stimulation further elevates glucose transport in virus-infected cells. NIH 3T3 cells were either mock infected or infected with wild-type polyoma virus and serum starved for 36 h. For serum-stimulated virus-infected cells, 15% calf serum was added 30 h after infection.

suggesting the possibility of a role of PI3P in vesicular trafficking.

Whether GLUT1 translocation fully accounts for increased 2DG transport in polyoma virus-infected cells remains to be determined. Other roles of PI 3-kinase may be envisaged. For example, 3-phosphoinositides might act in the plasma membrane as boundary lipids to directly regulate glucose transporter activity in a manner analogous to phospholipid effects on acetylcholine receptors, sarcoplasmic reticulum Ca^{2+}-ATPases, or potassium channels (53–57). Modulation of specific activity has indeed been demonstrated *in vivo* with adipocyte and pigeon erythrocyte GLUT1 (58, 59). Glucose transporter activity could also be indirectly regulated through calcium-independent protein kinase C isotypes which have been shown to be activated by more highly phosphorylated 3-phosphoinositides *in vitro* (60, 61). Finally, it is conceivable that the protein (serine/threonine) kinase activity intrinsic to PI 3-kinase (62) may be involved.

That PI 3-kinase-mediated increases in glucose uptake contribute to the tumor-inducing ability of polyoma virus is consistent with results of an earlier study showing that mutant 315YF induces tumors at a lower frequency and requiring more time to develop than those induced by wild-type virus (19). However, in addition to increases in glucose transport, PI 3-kinase binding to middle T also mediates activation of the ribosomal protein S6 kinase, pp70^{S6K} (J.D., unpublished results), and possibly other functions as well. Mutant 250YS, while retaining these PI 3-kinase-mediated functions, is nevertheless severely handicapped in transformation and tumorigenesis, indicating that persistent elevation of glucose uptake along with stimulation of S6 phosphorylation is not sufficient to induce complete neoplastic transformation. The downstream targets of the middle T-Shc pathway, including effector functions of activated Ras (10), are clearly essential for a fully transformed phenotype.

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